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Regions in β-Chemokine Receptors CCR5 and CCR2b That Determine HIV-1 Cofactor Specificity

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Summary

Macrophage-tropic (M-tropic) HIV-1 strains use the β-chemokine receptor CCR5, but not CCR2b, as a cofactor for membrane fusion and infection, while the dual-tropic strain 89.6 uses both. CCR5/2b chimeras and mutants were used to map regions of CCR5 important for cofactor function and specificity. M-tropic strains required either the amino-terminal domain or the first extracellular loop of CCR5. A CCR2b chimera containing the first 20 N-terminal residues of CCR5 supported M-tropic envelope protein fusion. Amino-terminal truncations of CCR5/CCR2b chimeras indicated that residues 2–5 are important for M-tropic viruses, while 89.6 is dependent on residues 6–9. The identification of multiple functionally important regions in CCR5, coupled with differences in how CCR5 is used by M- and dual-tropic viruses, suggests that interactions between HIV-1 and entry cofactors are conformationally complex.

Introduction

The entry of HIV-1 into target cells requires the participation of at least two cell surface molecules. All HIV-1 strains utilize CD4 as the primary virus receptor through a high affinity interaction with the viral envelope (env) protein. However, CD4 alone is not sufficient for virus entry: one or more additional cell surface molecules, termed cofactors, are required (Maddon et al., 1986; Ashorn et al., 1990; Chesebro et al., 1990; Dragic et al., 1992; Broder et al., 1993). Virus strains adapted for growth in transformed T cell lines (T-tropic) use the SDF-1 chemokine receptor as a cofactor (Feng et al., 1996). Expression of this protein, termed Fusin (originally termed 7TMS and also referred to as HUMSTR and LESTR), in conjunction with CD4 allows entry of T-tropic viruses into cells (Berson et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996). In addition, cells expressing both CD4 and Fusin form syncytia with cells expressing T-tropic env proteins. However, Fusin is not used as a cofactor by macrophage-tropic (M-tropic) isolates of HIV-1 (Berson et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996), which are far more prevalent than T-tropic virus strains, particularly during the asymptomatic period of infection (Tersmette et al., 1988, 1989; Schuitemaker et al., 1991, 1992; Conner et al., 1993).

The relationship of Fusin to the chemokine receptors, coupled with the observation that the β-chemokines RANTES, MIP-1α, and MIP-1β inhibit entry of M-tropic, but not T-tropic, virus strains (Cocchi et al., 1995; Paxton et al., 1996), led to the rapid identification of the β-chemokine receptor CCR5 as the major entry cofactor for primary, M-tropic virus strains (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996). CCR5, which is expressed in monocytes, macrophages, and primary T cells, binds to RANTES, MIP-1α, and MIP-1β (Alkhatib et al., 1996; Deng et al., 1996; Dragic et al., 1996; Samson et al., 1996). Expression of CCR5 in conjunction with CD4 in a variety of cell types renders them permissive for infection by M-tropic virus strains as well as for syncytia formation mediated by M-tropic env proteins (Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996). Other chemokine receptors can serve as entry cofactors for certain virus strains, including CCR3 and CCR2b (Choe et al., 1996; Doranz et al., 1996).

The identification of Fusin, CCR2b, CCR3, and CCR5 as entry cofactors for T- and M-tropic virus strains has important implications for understanding the molecular basis of viral tropism and affords new opportunities for designing antiviral strategies. It will be important to characterize regions of the chemokine receptors that are involved in chemokine binding and env protein interactions in order both to better understand the molecular basis of viral tropism and the molecular evolution of HIV-1 strains as they change cofactor usage, and to better design agents that might prevent cofactor utilization by primary HIV strains. In addition, structure–function studies of HIV-1 cofactors will be needed to understand the role these receptors play in viral entry and membrane fusion.

To identify regions in CCR5 that play a role in membrane fusion mediated by a variety of M-tropic and dual-tropic viruses, we constructed chimeric molecules based on CCR5 and CCR2b, the chemokine receptor to which CCR5 is most closely related (76% identity). We found that M-tropic viruses were most sensitive to changes in the very N-terminal portion of the amino-terminal domain and in the first extracellular loop. While substitution of either domain with the corresponding region of CCR2b was tolerated, substitution of both was

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not. We found several chimeric molecules that could function as cofactors for either M-tropic or dual-tropic env proteins, but not both. Our results identify regions in CCR5 required for cofactor function, indicate that interactions between CCR5 and M- and dual-tropic viruses can differ and are structurally complex, and suggest that the molecular evolution of virus strains for growth in a given target cell population may involve changes not only in the types of cofactors used, but also in how a given cofactor is utilized.

Results

The β-Chemokine Receptors CCR5 and CCR2b Support Fusion and Infection by Viruses with Distinct Tropisms

Introduction of CCR5 in conjunction with human CD4 into otherwise nonpermissive cells renders them fully permissive for M-tropic virus entry and for M-tropic env-mediated syncytia formation. By contrast, expression of CCR2b fails to render cells permissive for M-tropic env-mediated syncytia formation (Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996). However, the dual-tropic HIV-1 strain 89.6 was able to fuse with cells expressing CD4 and either CCR2b or CCR5 (Doranz et al., 1996). Therefore, to identify regions in CCR5 that are important for M-tropic strain cofactor function, we constructed a series of chimeric molecules between CCR5 and CCR2b. This approach had two important advantages. First, construction of chimeras between two closely related molecules is more likely to result in proper folding and transport to the cell surface (Doms et al., 1993). Second, since 89.6 uses both cofactors, we anticipated that CCR5/2b chimeras would likely function as cofactors for 89.6. Thus, 89.6 could serve as a positive control for cell surface expression.

Prior to generating chimeric molecules, we extended our earlier observations by testing the ability of CCR5 and CCR2b to function as cofactors for additional M-tropic viruses. To measure syncytia formation, we used a luciferase-based gene reporter assay (Doranz et al., 1996). In this assay, HeLa cells are infected with recombinant vaccinia virus vectors expressing the env protein of interest and the T7 RNA polymerase. Quail QT6 cells are transfected with plasmids encoding CD4, the desired cofactor, and the luciferase gene under control of the T7 promoter. QT6 cells were chosen as targets because expression of CD4 alone fails to render them permissive for env-mediated membrane fusion and because they are easily transfected. Fusion between the target and effector cells results in T7 polymerase-dependent luciferase expression, making this a sensitive and quantitative cell–cell fusion assay. We found that HeLa cells expressing the env proteins derived from the M-tropic strains JR-FL, ADA, and SF162, together with the clade E strain CM243, readily fused with QT6 cells expressing CD4 and CCR5 (Figure 1A). Fusion was not observed when either Fusin or CCR2b was used in place of CCR5. By contrast, the env protein derived from the dual-tropic virus strain 89.6 formed syncytia with cells expressing either Fusin, CCR2b, or CCR5 in conjunction with CD4, as previously reported (Doranz et al., 1996).

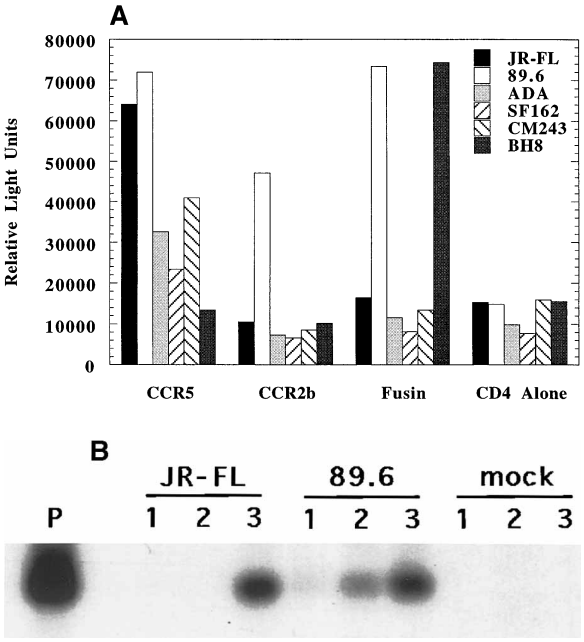

Figure 1. Ability of CCR5, CCR2b, and Fusin to Support env-Dependent Membrane Fusion

(A) QT6 cells expressing CD4, the indicated cofactor, and luciferase under control of the T7 promoter were mixed with HeLa cells infected with vaccinia virus vectors expressing either T-tropic (BH8), M-tropic (JR-FL, ADA, SF162, or the clade E virus CM243), or dual-tropic (89.6) env proteins. Effector cells were also infected with vTF1.1 (MOI = 10), which expresses T7 polymerase under the control of the vaccinia late promoter. Cells were allowed to fuse for 6–8 hr before lysis in Reporter Lysis Buffer (Promega) and assay for luciferase activity. Results are expressed in terms of relative light units (RLU).

(B) QT6 cells expressing CD4 alone (1) or with CCR2b (2) or CCR5 (3) were infected with HIV-1 strains JR-FL, 89.6, or mock supernatant. The following day, cells were lysed and HIV reverse transcription products were detected by PCR amplification of U3/U5 sequences followed by Southern blot. HIV-1 plasmid (P) served as a positive control.

We also tested the ability of CCR2b to function as a cofactor for virus infection. QT6 cells expressing CD4 alone or in combination with CCR5 or CCR2b were infected with either 89.6 or JR-FL. To detect virus entry 24 hr later, we used a PCR-based entry assay to detect early viral DNA transcripts. We found that CCR2b supported entry by 89.6 but not by JR-FL (Figure 1B). Thus CCR2b served as a fusion and infection cofactor for 89.6 but not for the M-tropic strains tested here.

The N-Terminus of CCR5 Is Sufficient but Not Necessary for JR-FL env-Mediated Fusion

To identify regions of CCR5 that are required for cofactor function, a series of chimeric molecules was generated in which individual extracellular domains of CCR2b were replaced with the corresponding regions of CCR5. In this way, we could determine whether any single region of CCR5 could confer M-tropic cofactor activity to CCR2b. Chimeric molecules were created by utilizing common restriction sites in regions conserved between

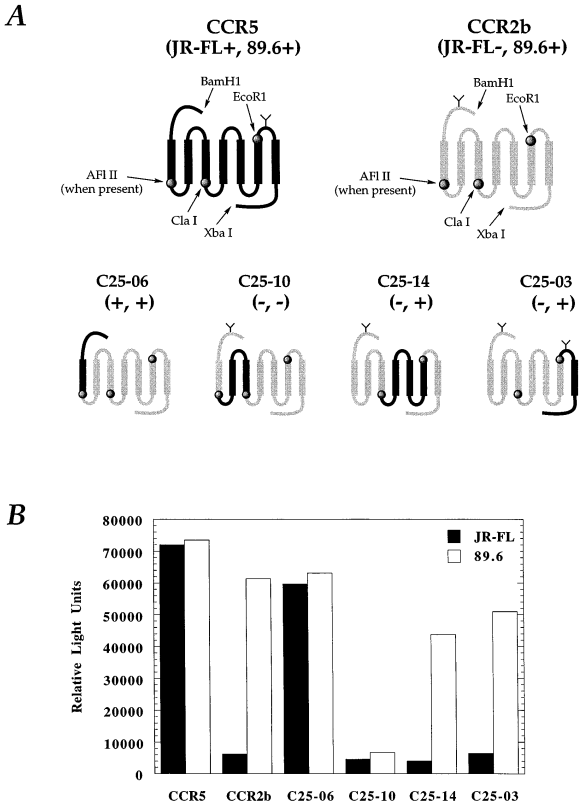

Figure 2. CCR2b Chimeras with Single CCR5 Domain Replacements

(A) The structures of CCR5, CCR2b, and chimeras containing single-domain swaps of CCR5 into CCR2b are represented schematically. The junctions between chimeric segments correspond to the AflII, ClaI, and EcoRI sites represented in the parental structures. The putative N-linked glycosylation sites originating from CCR2b and CCR5 are represented when present in the constructs. The phenotype for each construct with regards to enabling fusion for JR-FL and 89.6, respectively, is shown for convenience above each construct in parenthesis. (+,+) indicates that the molecule supported fusion by both env proteins, while (−,+) indicates fusion for 89.6 alone.

(B) QT6 cells expressing CD4, the indicated cofactor, and luciferase under control of the T7 promoter were mixed with HeLa cells expressing the T7 polymerase and the indicated env protein. The degree of cell–cell fusion was determined by measuring relative light units 8 hr after mixing, as in Figure 1.

the two molecules (Figure 2A). This approach also resulted in substitution of CCR2b transmembrane and cytoplasmic domains with those from CCR5. However, as shown in Figure 8, the transmembrane domains of CCR5 and CCR2b are highly conserved (89% identity, including all seven proline residues), as are the three intracellular loops (92% identity). Including highly conservative amino acid substitutions, the degree of similarity between the transmembrane and intracellular loops of CCR5 and CCR2b is 95% and 96%, respectively. By contrast, the extracellular domains of CCR5 and CCR2b share only 45% amino acid identity. Thus the amino-terminal domain and extracellular loops are likely to play the major role in determining the cofactor phenotypes exhibited by the chimeric molecules.

The chimeras, depicted in Figure 2A, were expressed with CD4 in QT6 cells and then mixed with HeLa cells expressing either the 89.6 or the JR-FL env proteins. As shown in Figure 2B, substitution of the amino-terminal domain and first transmembrane segment of CCR2b with the corresponding region of CCR5 resulted in a chimera that supported fusion by both the JR-FL and 89.6 env proteins. We also note that this chimera (C25-06) has no potential N-linked glycosylation sites, indicating that N-linked glycosylation is not required for cofactor function. Indeed, elimination of the N-linked glycosylation site in CCR5 by site-directed mutagenesis had no effect on cofactor function (data not shown). Replacement of the second or third extracellular loops of CCR2b with those from CCR5 failed to support JR-FL mediated membrane fusion. However, both of these chimeras (C25-14 and C25-03) supported fusion mediated by 89.6, indicating that they were expressed on the cell surface. One chimera, C25-10, failed to support fusion by either JR-FL or 89.6. Since the 89.6 env protein can fuse with cells expressing CD4 and either CCR5 or CCR2b, we think it most likely that this chimera is not expressed at the cell surface, perhaps due to a folding defect. Development of specific antisera will make it possible to measure surface expression of this and other chimeric molecules.

The results with the first set of chimeric molecules indicated that the amino terminus of CCR5, when introduced into a CCR2b background, was sufficient to confer M-tropic cofactor activity to CCR2b. To determine whether the amino-terminal domain of CCR5 was required for cofactor activity, we sequentially replaced each extracellular domain of CCR5 with the corresponding region of CCR2b (Figure 3A). Somewhat surprisingly, we found that no single extracellular domain of CCR5 is required for cofactor function when replaced with the homologous domain from the CCR2b receptor (Figure 3B). However, the amino-terminal domain appears to play an important role in cofactor function as it is sufficient to confer M-tropic cofactor activity to CCR2b. That it is not absolutely required for cofactor function indicates that one or more additional domains of CCR5 play an important role in virus entry and membrane fusion.

N-Terminal Domain Truncations Differentiate 89.6 and JR-FL Cofactor Usage

To further delineate the region of the CCR5 amino terminus that is critical for cofactor function, we constructed a series of CCR5 molecules containing sequential four–amino acid deletions after the N-terminal methionine (Figure 3A). We found that elimination of the first eight residues from the mature molecule (i.e., after the initiator methionine) had no effect on JR-FL or 89.6 env-mediated syncytia formation (Figure 3B). However, removal of an additional four or eight residues blocked fusion by 89.6 but not by JR-FL. Thus 89.6 is more sensitive to truncations in the amino-terminal domain of CCR5 than is the M-tropic strain JR-FL, indicating that different env proteins can utilize the same cofactor in different ways.

Since regions of CCR5 other than the N-terminal domain can also allow fusion by JR-FL (Figure 3), we reasoned that truncations of the CCR5 amino terminus

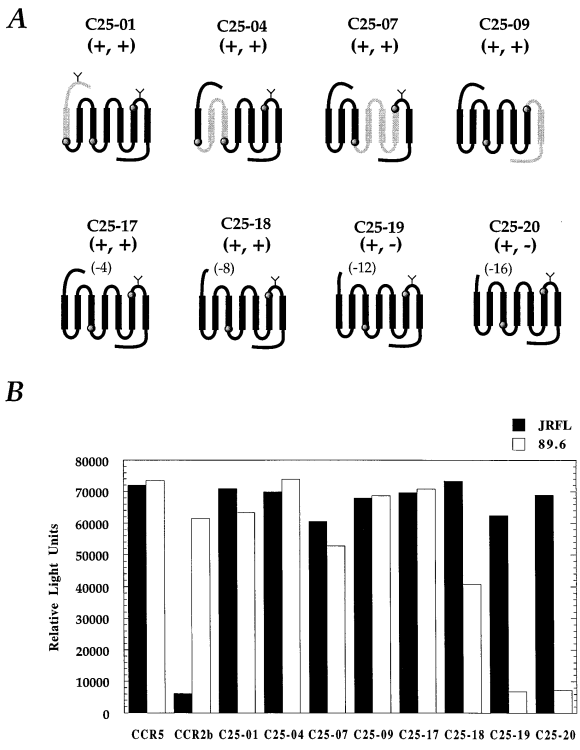

Figure 3. CCR5 Chimeras with Single CCR2b Domain Replacements and N-Terminal CCR5 Truncations

(A) The structures of four chimeric molecules containing single-domain exchanges of CCR2b (light shading) into CCR5 (dark shading) are shown in the top row, and a series of N-terminal CCR5 deletions are shown in the second row, in which 4, 8, 12, or 16 residues were deleted, respectively. The ability of each construct to support fusion for JRFL and 89.6, respectively, is shown above each construct in parentheses.

(B) Fusion of QT6 cells expressing CD4 and the indicated cofactor or chimera with HeLa cells expressing the JR-FL or 89.6 env proteins was determined using the luciferase reporter assay as shown in Figures 1 and 2. Fusion results with CCR5 and CCR2b are included for reference.

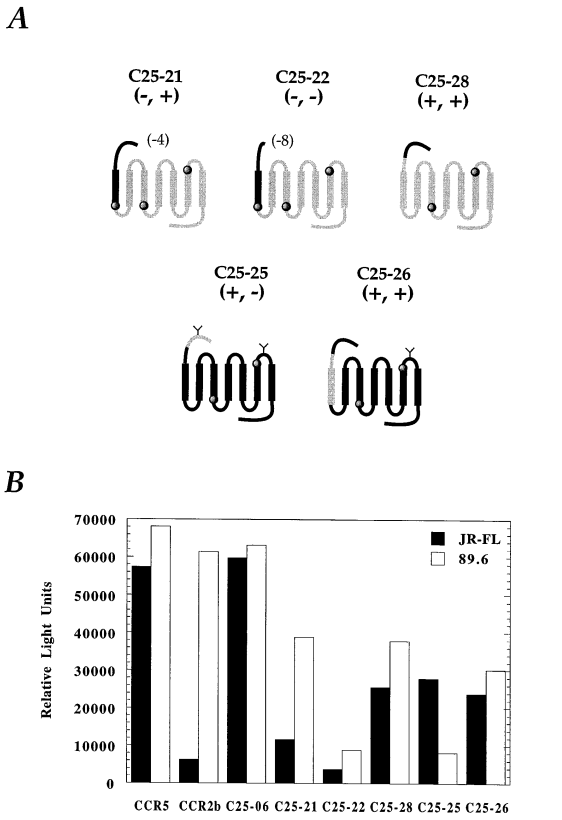

Figure 4. Truncated CCR5 Amino-Terminal Domain Chimeras

(A) The following chimeric molecules are depicted: C25-21, lacking the first four residues of the CCR5 amino terminus; C25-22, lacking the first eight residues of CCR5; C25-28, in which the first 20 residues of CCR5 are in a CCR2b background; C25-25, in which the first 20 residues of CCR2b are in a CCR5 background; and C25-26, in which the first 20 residues of CCR5 are followed by the rest of the CCR2b amino-terminal domain, the first transmembrane domain of CCR2b, and the rest of CCR5.

(B) Fusion of QT6 cells expressing CD4 and the indicated cofactor or chimeric molecules with HeLa cells expressing the JR-FL or 89.6 env proteins was determined using the luciferase reporter assay. Fusion results obtained with CCR5, CCR2b, and C25-06 are included for reference.

might not reveal its true role in cofactor function if assayed on its normal, functionally redundant background. We therefore constructed a series of chimeric molecules in which the truncated amino-terminal domains of CCR5 were placed into a CCR2b background (Figure 4A). Chimera C25-21, which contains the N-terminal domain of CCR5 truncated by four residues in a CCR2b background, supported fusion by 89.6 but not by JR-FL (Figure 4B). This was somewhat surprising, since the identical truncation in a CCR5 background (C25-17) supported fusion by both JR-FL and 89.6. Removal of an additional four residues (C25-22) abolished cofactor function for both 89.6 and JR-FL, even though the identical truncation in a CCR5 background (C25-18) supported fusion by both viral env proteins. While it is possible that chimera C25-22 may not be expressed on the cell surface, we note that the truncation itself is tolerated (C25-18), making it more likely that C25-22 is delivered to the plasma membrane. Therefore, for both JR-FL and 89.6, N-terminal truncations that otherwise have no effect on cofactor function in CCR5 actually prevent cofactor usage in a CCR2b background. These findings indicate that regions other than the amino terminus of CCR5 play important roles in governing cofactor function, that the ability of JR-FL to utilize the CCR5 amino-terminal domain is dependent on amino acids 2–5, and that the ability of 89.6 to utilize the CCR5 amino-terminal domain is dependent upon residues 6–9 of CCR5. Further truncations of the CCR5 N-terminus on a CCR2b background did not give rise to active cofactors (data not shown).

To identify the minimal portion of the CCR5 amino terminus capable of conferring cofactor function to CCR2b, three additional chimeras were constructed (Figure 4A). We found that substitution of the first 20 residues of CCR2b with those from CCR5 (C25-28) supported fusion by both JR-FL and 89.6, while the reciprocal chimera containing the first 20 residues of CCR2b in a CCR5 background (C25-25) supported fusion by JR-FL alone (Figure 4B). The inability of C25-25 to support fusion by 89.6 was surprising, since chimera C25-01, which contained the entire CCR2b amino terminus and first transmembrane domain in a CCR5 background,

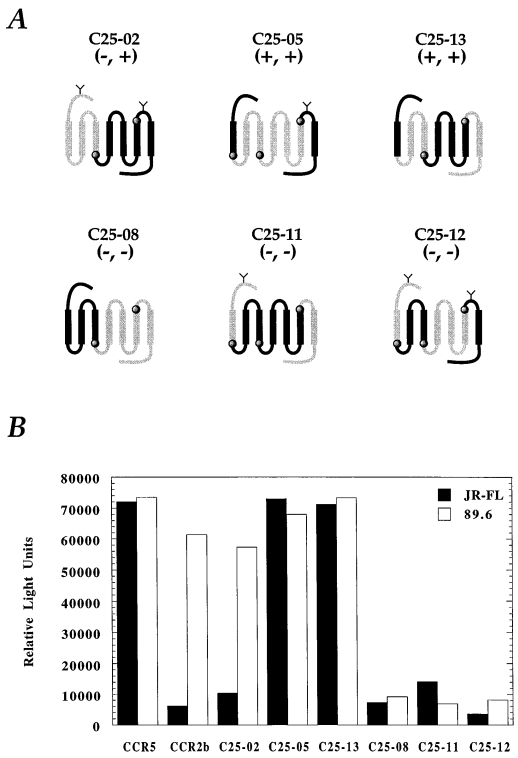

Figure 5. Chimeras with Multiple Domain Substitutions

(A) The structures of six chimeras containing multiple domain substitutions are depicted, along with their ability to support fusion by JR-FL and 89.6.

(B) Fusion of QT6 cells expressing CD4 and the indicated cofactor or chimera with HeLa cells expressing the JR-FL or 89.6 env proteins was determined using the luciferase reporter assay. Fusion results with CCR5 and CCR2b are included for reference.

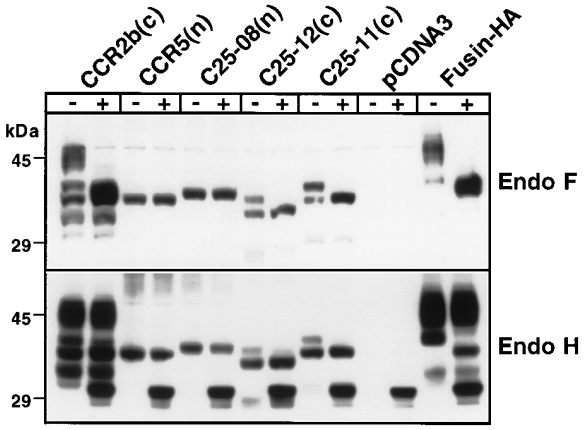

Figure 6. N-Linked Carbohydrate Processing of CCR2b, CCR5, and Nonfunctional Chimeras

QT6 or BSC-1 cells expressing the indicated receptors or chimeras containing the HA antigenic tag on either the amino terminus (n) or carboxyl terminus (c) were lysed and processed for endoglycosidase F and H digestion as in Experimental Procedures. The reactions were then subjected to SDS–PAGE in the presence of 4 M urea, transferred to PVDF, and Western blotted with the MAb 12CA5, which detects the HA-tag. The top panel shows endo F digestion, and the bottom panel shows endo H digestion. (−), mock-treated sample; (+), enzyme-treated sample. The mobilities of the indicated molecular weight standards are shown on the left.

did support 89.6 fusion. These results show that, while complete substitution of the amino-terminal domain of CCR5 with that from CCR2b is tolerated by both viruses, partial domain substitutions within the amino-terminal domain sometimes (C25-25), but not always (C25-26), fail to support fusion. Partial domain substitutions may alter the conformation of the amino-terminal domain or perhaps affect the way in which it interacts with the extracellular loops.

The Role of Extracellular Loop Domains in Cofactor Function

The chimeras described above implicated both the amino terminus of CCR5 as well as one or more additional domains as playing important roles in cofactor function. In order to assess the contribution of other domains in CCR5 to cofactor function, chimeras containing multiple domain substitutions were constructed (Figure 5A). As shown in Figure 3B, we found that substitution of the CCR5 amino-terminal domain with that from CCR2b did not affect cofactor function, even though several chimeric and mutant molecules (C25-06, C25-19, C25-20, C25-21) clearly showed that the amino-terminal domain of CCR5 plays an important role in supporting env-mediated membrane fusion. However, we found that substitution of both the amino-terminal and the first extracellular loops of CCR5 with the homologous CCR2b domains (C25-02) supported fusion by 89.6 but not by JR-FL. This result indicated that the first extracellular loop, in conjunction with the amino-terminal domain, plays an important role in governing M-tropic cofactor function. While JR-FL can tolerate substitution of either the amino-terminal domain or first extracellular loop of CCR5 with the homologous CCR2b regions, it cannot tolerate substitution of both. Thus, in the presence of the CCR5 amino-terminal domain, multiple changes in the first, second, and third extracellular loops are well tolerated (C25-05, C25-13).

Several chimeric molecules failed to support fusion by either 89.6 or JR-FL, including C25-08, which contained both the amino-terminal domain and the first extracellular loop of CCR5. To determine whether these chimeric molecules were processed normally, we assessed their glycosylation states. An antigenic tag corresponding to a sequence nine amino acids long and derived from influenza hemagglutinin (HA) was placed at the amino or carboxyl terminus of CCR5, CCR2b, and three chimeric molecules that failed to support membrane fusion: C25-08, C25-12, and C25-11. The tagged versions of CCR5 and CCR2b supported membrane fusion by 89.6, indicating that they are transported to the cell surface. QT6 cells expressing the indicated receptor were lysed, and aliquots were digested with endoglycosidase F (endo F) to remove N-linked carbohydrate chains. Fusin-HA was also examined, since we have previously shown that it contains at least one N-linked carbohydrate chain (Berson et al., 1996). We found that CCR2b was N-glycosylated (Figure 6), indicating that the single N-linked consensus site in the CCR2b amino-terminal domain is utilized. However, digestion of CCR5 with endo F failed to increase its mobility in SDS–PAGE, indicating that it

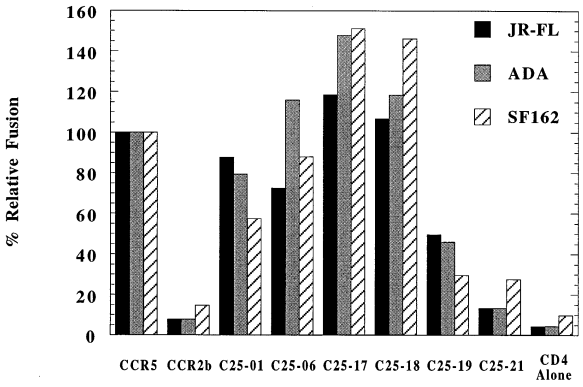

Figure 7. Different M-Tropic env Proteins Utilize CCR5/2b Chimeras in the Same Way

QT6 cells expressing CD4 and the indicated wild-type or chimeric cofactor were mixed with HeLa cells expressing the indicated M-tropic env protein. Fusion was determined as in the previous figures.

is not N-glycosylated. Chimeric molecules containing the CCR2b amino-terminal domain were N-glycosylated as well. Migration of chimera C25-08 was not affected by endo F digestion, consistent with the fact that it does not contain N-linked consensus sequences.

To determine whether the glycosylated receptors were processed normally, we subjected aliquots of cell lysates to digestion with endoglycosidase H (endo H). We found that both Fusin and CCR2b were largely resistant to endo H digestion, indicating that both receptors were transported through the Golgi apparatus. However, chimeras C25-12 and C25-11 were completely or largely sensitive to endo H digestion, suggesting that these molecules were not transported to the Golgi and cell surface. Thus, the failure of C25-12 and C25-11 to support fusion by HIV-1 env proteins can be attributed to a transport defect. It is interesting to note that, of the five chimeric molecules in this study that failed to function as entry cofactors, four contained the second extracellular loop of CCR2b, a region of the receptor that contains an unpaired Cys residue not present in CCR5. Since unpaired Cys residues often form inappropriate disulfide bonds in the ER (Doms et al., 1993), chimeric receptors containing the second extracellular loop of CCR2b might be at greater risk for misfolding and retention in the ER.

Clade B M-Tropic Strains Utilize Similar CCR5 Domains

Our studies indicated that the M-tropic env protein derived from JR-FL and the dual-tropic 89.6 env protein exhibited differences in their abilities to utilize chimeric and mutant CCR5 molecules. To determine whether virus strains with the same tropism also exhibited differences in their use of CCR5, two additional M-tropic viruses were tested for their abilities to utilize a subset of the chimeric molecules, including C25-01, C25-06, C25-21, C25-17, C25-18, and C25-19. As shown in Figure 7, no differences were observed between the different virus strains, indicating that the M-tropic viruses JR-FL, ADA, and SF162 interact with CCR5 in an identical or very similar fashion, but one that is distinct from the dual-tropic 89.6 strain.

Discussion

The entry of HIV-1 into target cells involves interactions with the primary virus receptor, CD4, and one or more cofactors (Broder et al., 1993; Alkhatib et al., 1996; Berson et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996). The specific type of cofactor used is a major determinant of viral tropism. Thus, HIV-1 strains adapted for growth on transformed T cell lines use a seven-transmembrane domain receptor, Fusin (Berson et al., 1996; Choe et al., 1996; Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996), that is expressed at high levels in T cell lines (Federsppiel et al., 1993; Loetscher et al., 1994). While some primary virus isolates also exhibit the capability of infecting transformed T cell lines, such T-tropic viruses do not appear to play a significant role in virus transmission and often cannot be recovered from HIV-1 positive individuals. Instead, most primary virus isolates fail to infect transformed T cell lines, though they infect primary macrophages and primary T cells efficiently. These M-tropic virus strains are responsible for sexual transmission of HIV-1 and are the prevalent virus type isolated after seroconversion and during the asymptomatic portion of the disease (Åsjö et al., 1986; Tersmette et al., 1988, 1989; Schuitemaker et al., 1991, 1992; Roos et al., 1992; Conner et al., 1993). With time, both dual-tropic and T-tropic viruses emerge in some individuals, with dual-tropic viruses perhaps representing an intermediate phenotype (Tersmette et al., 1988; Collman et al., 1992).

Recently, CCR5, a chemokine receptor that binds RANTES, MIP-1α, and MIP-1β (Samson et al., 1996), has been shown to be the principle M-tropic virus entry cofactor (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996). The identification of CCR5 as the major entry cofactor for M-tropic viruses was aided by its homology ($\sim$30%) to Fusin and by observations that RANTES, MIP-1α, and MIP-1β are the major viral suppressive factors secreted by $CD8^+$ cells (Cocchi et al., 1995) and that lymphocytes from some exposed, uninfected individuals secrete high levels of these chemokines (Dragic, 1996; Paxton et al., 1996). Most M-tropic virus strains studied thus far appear to exclusively use CCR5 as an entry cofactor, though the number of virus strains for which cofactor usage has been studied is small, especially viruses from other clades. It is clear, however, that some virus isolates can use alternative chemokine receptors as entry cofactors (Choe et al., 1996; Doranz et al., 1996). The dual-tropic strain 89.6 is remarkably promiscuous: in addition to CCR5, it can use Fusin and the chemokine receptors CCR3 and CCR2b as entry cofactors (Doranz et al., 1996), suggesting that interactions between viruses and chemokine receptors will be dependent upon conserved, conformational elements.

Identifying regions in CCR5 and other viral cofactors that are important for viral entry will be important for understanding the molecular basis of viral tropism and

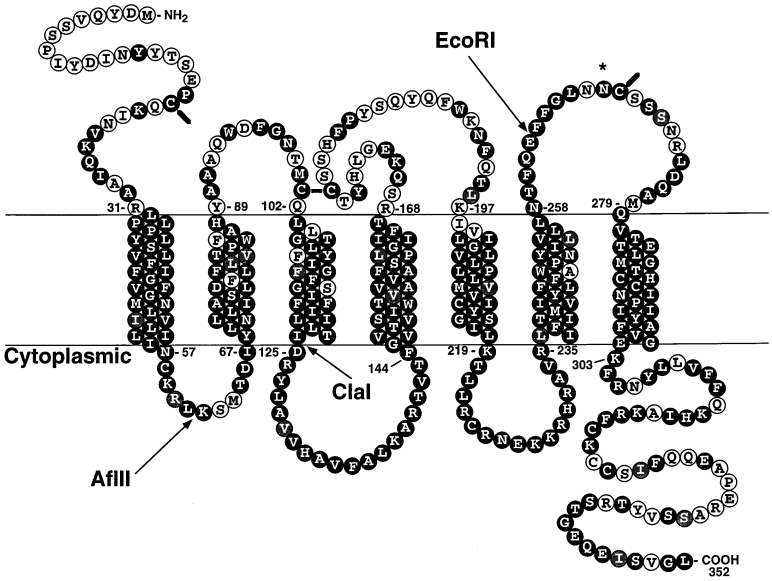

Figure 8. Proposed Membrane Topography of CCR5

The amino acid sequence of CCR5 is depicted. Extracellular Cys residues are indicated by bars, and the single N-linked glycosylation consensus site is indicated by an asterisk. Residues that are identical to those in CCR2b are indicated by dark shading, while highly conservative substitutions are indicated by light shading. The locations of the restriction sites used to generate the chimeric molecules are shown.

may assist in the design of novel antiviral compounds. The approach we took to identify functionally important CCR5 domains involved the construction of chimeras between CCR5 and CCR2b and mutagenesis of the CCR5 amino-terminal domain. CCR2b is expressed in monocytes, binds the CC chemokines MCP-1 and MCP-3 (Charo et al., 1994; Franci et al., 1995), and shares 76% amino acid identity with CCR5. Due to their structural similarity, we anticipated that a high proportion of chimeras generated between these two molecules would be transported to the cell surface. In fact, at least 18 of the 24 chimeric and mutant molecules studied here were expressed on the cell surface as judged by their ability to support env-mediated membrane fusion. A further advantage of using CCR2b as a template for studying functionally important CCR5 domains is that it is used by 89.6 as an entry cofactor, making it likely that most chimeric molecules would function as cofactors for this viral env protein. We found four chimeric or mutant receptors that functioned for 89.6 but not for JR-FL and three that functioned for JR-FL but not for 89.6. Thus we were able to detect functional cofactors that would have been scored as negative had we used only one type of env protein. Finally, the transmembrane and cytoplasmic loops of CCR5 and CCR2b are highly conserved (95% and 96% including highly conservative substitutions, respectively), whereas the extracellular domains share only 45% amino acid identity (Figure 8). Thus the extracellular domains are likely to play the major role in determining the cofactor phenotypes exhibited by the chimeric molecules.

Our results indicate that the amino terminus of CCR5 plays an important role in virus entry. The amino-terminal domain of CCR5 was the only region that, when introduced into CCR2b, conferred M-tropic cofactor activity to the resulting chimera. Further analysis showed that the first 20 residues of CCR5, the region of CCR5 that is most highly divergent from CCR2b (Figure 8), were sufficient for conferring M-tropic cofactor activity to CCR2b. However, other regions of CCR5 must also be important for cofactor activity, since the N-terminal domain of CCR5 could be replaced with the corresponding region from CCR2b and still function as an M-tropic cofactor. As a consequence, changes in the CCR5 amino-terminal domain that might otherwise affect cofactor function might go unobserved when assayed on the functionally redundant CCR5 background. Indeed, additional complexities were observed when CCR5 amino-terminal mutations were examined in a CCR2b background. Thus the ability of M-tropic env proteins to use CCR5 was dependent on residues 2–5 of the N-terminal domain, while 89.6 was strongly dependent on residues 6–9. Therefore, the amino-terminal domain of CCR5 plays an important role in cofactor function for both M- and dual-tropic viruses, though there are differences in how each virus type utilizes this domain.

Our results also implicate the first extracellular loop of CCR5 as being important for cofactor function, although like the amino-terminal domain it is not absolutely necessary: the first loop of CCR5 can be replaced by the corresponding region in CCR2b without loss of activity. The 14-residue-long first extracellular loop contains a conserved Cys residue thought to form a disulfide bond with a Cys residue in the second extracellular loop, along with three charged residues, none of which are conserved between CCR5 and CCR2b. Chimera 25-02 is the most informative with regards to the potential function of this domain. Replacement of both the amino-terminal domain and the first extracellular loop (C25-02) resulted in a chimeric molecule that could be used by 89.6 but not by M-tropic viruses as a fusion cofactor. By contrast, double substitution of the first and second loops (chimera C25-05) or the first and third loops (C25-13) of CCR5 with those from CCR2b was tolerated, as long as the CCR5 amino-terminal domain was present. We found that M-tropic viruses could tolerate substitution of either the N-terminal domain or the first extracellular loop of CCR5 with the corresponding CCR2b domains but not of both simultaneously.

We also constructed chimeras to test the role of the

second and third extracellular loops in cofactor function. The second extracellular loop is highly divergent between CCR5 and CCR2b, with only 11 of 30 residues being conserved, including a Cys residue and three sequential charged amino acids. Introduction of the second loop from CCR5 into CCR2b failed to confer M-tropic cofactor function, while substitution of the second loop of CCR5 with that from CCR2b failed to prevent M-tropic env-mediated membrane fusion. Thus, the second loop appears to play little role in cofactor specificity, at least when tested in either a CCR5 or CCR2b background. However, we cannot rule out the possibility that residues conserved between CCR5 and CCR2b participate in some way.

The third extracellular loop is the most highly conserved ectodomain region between CCR5 and CCR2b, with 17 of 23 residues being identical, including all four charged residues. Since introduction of the third extracellular loop of CCR5 into a CCR2b background was not sufficient for M-tropic cofactor activity (C25-03), these conserved residues by themselves are clearly not sufficient for cofactor function. However, due to their similarity, the fact that replacement of CCR5's third loop with that from CCR2b had no effect on cofactor function should not be taken as evidence that this region is not important. Site-directed mutagenesis or construction of chimeras between more distantly related molecules will be required to more fully understand the role that the third extracellular loop plays in virus entry. In fact, the high degree of conservation of this region, the use of both receptors as cofactors by 89.6 (Doranz et al., 1996), and studies with the IL8 receptor (Hebert et al., 1993; Horuk, 1994) all suggest that residues in the third loop may help regulate cofactor function.

Relatively little is known about the structure–function relationships of chemokine receptors and their ligands. However, a predominant theme emerging from studies in this area is that the N-terminus of these molecules plays a major role in specificity and function. The amino terminus of the Duffy antigen, a receptor that binds both CC and CXC chemokines, plays a critical role in ligand binding (Zhao-hai et al., 1995), and the N-terminal region of the IL8 receptor is a major determinant in defining ligand specificity (Hebert et al., 1993; Horuk, 1994; Ahuja et al., 1996). Alanine-scanning mutagenesis of IL8-RA identified three charged residues important for ligand binding, including two in the third extracellular loop of the receptor (Hebert et al., 1993). The amino-terminal domains of Fusin (Feng et al., 1996) and CCR5 also appear to be important for HIV env fusion activity. However, as is the case for the IL8 receptor, our findings indicate that multiple regions of CCR5 are important for HIV env cofactor activity, including the N-terminus and first extracellular loop. The involvement of multiple extracellular domains of CCR5 in virus entry is not surprising. By analogy with other seven transmembrane domain receptors, conserved disulfide bonds (Figure 8) are likely to bring the extracellular domains of CCR5 in close proximity to one another (Horuk, 1994; Murphy, 1994).

We have shown a distinction between M- and dual-tropic HIV virus strains in their use of CCR5 as a fusion cofactor. Studies with several chemokines have shown that relatively subtle changes in chemokine structure can result in a different receptor-binding profile. Substitution of Leu-25 in IL8 with Tyr enables this CXC chemokine to bind to the CC chemokine receptor CCR1 (Lusti-Narasimhan et al., 1996; Wells et al., 1996), while deletion of the RANTES amino terminus enables it to bind to multiple chemokine receptors (Gong et al., 1996). Although the identification of multiple functionally important regions in CCR5 for cofactor activity suggests that structurally complex interactions may occur between CCR5 and M-tropic viruses, relatively minor changes in env structure could result in altered coreceptor utilization that would be important for virus evolution. Further mutagenesis studies will aid in better defining the critical determinants for cofactor activity and assist in the design of novel antiviral compounds that may be capable of blocking both M- and dual-tropic isolates.

Experimental Procedures

CCR5/CCR2b Hybrid Constructs

The CCR5 construct has been described previously (Samson et al., 1996). The coding region of CCR2b was amplified by PCR using NTerm2 (5′-TCGAGGATCCACAACATGCTGTCCACA-3′) and Cterm2 (5′-CTGATCTAGACCTCGTTTTATAAACCAG-3′) as primers and cloned between the BamHI and XbaI sites of pcDNA3. The AflII restriction site was introduced by site-directed mutagenesis in a region corresponding to the end of the first transmembrane region (Figure 8). Mutagenesis was done by PCR using primers Nterm5 and Afl2R5 (5′-AGTCATGCTCTTAAGCCTTTTGCAG-3′) as well as Afl2F5 (5′-CTGCAAAAGGCTTAAGAGCATGACT-3′) and Cla1R (5′-AGCCAGGTACCTATCGATTGTCAG-3′) for CCR5, and Nterm2 and Afl2R2 (5′-AGTCAAGCACTTAAGCTTTTTGCAG-3′) as well as Afl2F2 (5′-CTGCAAAAAGCTTAAGTGCTTGACT-3′) and Cla1R for CCR2b. Products were cleaved by either BamHI and AflII or AflII and ClaI and were cloned by trimolecular ligation in pcDNA3-CCR5 or in pcDNA-CCR2b cleaved with BamHI and ClaI. All constructions involving PCR were verified by sequencing. The various hybrid combinations (C25-01 to C25-14) were performed by transferring restriction fragments flanked by the common BamHI, AflII, ClaI, EcoRI, and XbaI sites between the CCR5 and CCR2b constructs.

Deletion Mutants

The deletion mutants of the CCR5 N-terminal extracellular region (C25-17 to C25-20) were constructed by generating PCR fragments, using Afl2R5 as common reverse primer and N5D4 (5′-TCGAGGATC CAAGATGTCAAGTCCAATCTAT-3′), N5D8 (5′-TCGAGGATCCAAGA TGTATGACATCAATTAT-3′), N5D12 (5′-TCGAGGATCCAAGATGTAT TATACATCGGAG-3′), and N5D16 (5′-TCGAGGATCCAAGATGGAGC CCTGCCAAAAA-3′), respectively, as forward primers. The PCR products were cleaved with BamHI and AflII and cloned into the CCR5 construct cleaved with the same enzymes. Amplified segments were verified by sequencing. The deleted CCR5 N-terminal segments were transferred onto the CCR2b background (C25-21 to C25-24) by cloning BamHI/AflII fragments into the pcDNA3-CCR2b construct.

Other Constructs

N- and C-terminally tagged constructs were made by PCR incorporation of the 12CA5 epitope of the influenza hemagglutinin protein (HA). N-terminal tagging of CCR5 involved the use of the NTag5 forward primer (5′- TCGAGGATCCAAGATGTACCCCTACGACGTG CCCGACTACGCCGGGCCCGGGGATTATCAAGTGTCA-3′), inserting the peptide YPYDVPDYAGPG immediately after the initial methionine, and the Afl2R5 reverse primer. The PCR product was cleaved by EcoRI and AflII and cloned into the pcDNA3-CCR5 construct. The HA tag was also introduced at the C-terminus of both CCR5 and CCR2b, using a similar approach.

The CCR5/CCR2b N-terminal hybrids (C25-26 and C25-28) were constructed using the Nterm5 and N5>2R1 (5′-TCAAATTTATGACA

GGGCTCCGATGTATA-3′) primers and N5>2F1 (5′-TATACATCGGA GCCCTGTCATAAATTTGA-3′) and Afl2R2 primers, respectively, in a first PCR experiment. The combined products were then amplified with Nterm5 and Afl2R2 alone, cleaved by BamHI and AflII, and cloned into pcDNA3-CCR5 and pcDNA3-CCR2b. The CCR2b/CCR5 N-terminal hybrid C25-25 was constructed in a similar fashion. The pREP8-Fusin (Lester) and pCDNA3-CCR5 construct have been previously described (Berson et al., 1996; Doranz et al., 1996). The luciferase-T7 plasmid was obtained from Promega. The plasmid pT4, providing expression of CD4 under the control of the CMV promoter, was provided by Dr. Dennis Kolson (University of Pennsylvania). All plasmids were prepared for the various constructs by using Qiagen Maxi-prep kits.

Cells and Viruses

HeLa and QT6 cells were cultured as previously described (Doranz et al., 1996). Vaccinia viruses encoding the envs of a variety of HIV-1 strains included vSC60 (BH8), vCB39 (ADA), vCB28 (JR-FL), vCB32 (SF162), and vCB53 (CM243, an M-tropic primary clade E virus) (Broder and Berger, 1995). We also used the recombinant virus vTF1.1, encoding the T7 RNA polymerase (Alexander et al., 1992). vBD3, expressing the 89.6 env protein, was described previously (Doranz et al., 1996). Infection studies using PCR primers to amplify a 430 bp region of U3/U5 LTR DNA sequences were performed as previously described (Doranz et al., 1996).

Gene Reporter Fusion Assay

To quantitate cell–cell fusion events, we used a luciferase-based gene reporter assay. T7 RNA polymerase and env proteins were introduced into effector HeLa cells by recombinant vaccinia viruses. Target QT6 cells were transfected with CD4, luciferase-T7, and accessory factor constructs as indicated in the text and legends. Vaccinia-encoded proteins were produced by infecting cells at a multiplicity of infection of 10 for 1.5–4 hr at 37°C. Effector cells were then trypsinized, washed with PBS, resuspended in media, and incubated at 32°C overnight in the presence of rifampicin. Proteins were generally introduced into target QT6 cells in 24-well plates by transfection of 2 μg of each plasmid using calcium phosphate precipitation. To initiate fusion, target and effector cells were mixed in 24-well plates at 37°C in the presence of ara-C and rifampicin. After 8–10 hr of fusion, 2–3 $\times$ 10^5 cells were lysed in 150 μl of reporter lysis buffer (Promega) and assayed for luciferase activity according to the manufacturer's instructions (Promega).

Glycosylation Studies

HA-tagged receptors were expressed for 24 hr in QT6 cells using the vaccinia virus T7 polymerase system (Earl and Moss, 1991). The cells were washed with PBS and lysed in 0.1 M Tris (pH 8), 0.1 M NaCl, 1 mM $CaCl_2$, and 1% Triton X-100 (TX-100) for 30 min at 4°C. The LESTR-HA lysate was prepared as above from BSC-1 cells infected with vBD4 (Berson et al., 1996). For endo F digestion, lysates were diluted 1:1 with 0.2 M phosphate (pH 7.9), 0.1% sodium dodecyl sulfate (SDS), and 0.1% β-mercaptoethanol and boiled for 5 min. An equal volume of the above buffer was added, the reaction was brought to 1% TX-100, and 15 mU/μl N-glycosidase F (Boehringer Mannheim) was added. The reaction was incubated overnight at 37°C and stopped with SDS–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer containing 4 M urea. For endo H digestion, lysates were diluted 1:4 with 60 mM phosphate (pH 5.7) and 1% SDS and boiled for 5 min. 55 μU/μl of endo H (Boehringer Mannheim) was added and the reactions incubated overnight at 37°C. The reaction was stopped with SDS-PAGE sample buffer containing 4 M urea. Endo F and endo H samples were analyzed by SDS–PAGE gels containing 4 M urea, and the gel was transferred onto PVDF (Millipore), blocked with Blotto (PBS with 0.1% Tween-20, 5% dried milk) and incubated with the murine MAb 12CA5 directed to the HA tag in Blotto overnight at 4°C. Bound antibody was detected by incubation with goat-anti-mouse HRP (Boehringer-Mannheim) diluted 1:20,000 in Blotto for 25 min at room temperature followed by chemilumenescent detection of HRP (ECL, Amersham).

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